Enhancement of Antibody Detection in Cancer Using Panel of Recombinant Tumor-associated Antigens

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Abstract
Cancer sera contain antibodies which react with a unique group of autologous cellular antigens called tumor-associated antigens (TAAs). This study determines whether a mini-array of multiple TAAs would enhance antibody detection and be a useful approach to cancer detection and diagnosis. The mini-array of TAAs comprised full-length recombinant proteins expressed from cDNAs encoding c-myc, p53, cyclin B1, p62, Koc, IMP1, and survivin. Enzyme immunoassay was used to detect antibodies in 527 sera from six different types of cancer. Antibody frequency to any individual TAA was variable but rarely exceeded 15–20%. With the successive addition of TAAs to a final total of seven antigens, there was a stepwise increase of positive antibody reactions up to a range of 44–68%. Breast, lung, and prostate cancer patients showed separate and distinct profiles of reactivity, suggesting that uniquely constituted antigen mini-arrays might be developed to distinguish between some types of cancer. Distinct antibody profiles were not observed in gastric, colorectal, and hepatocellular carcinomas with this set of seven TAAs. Detection of autoantibodies in cancer can be enhanced by using a mini-array of several TAAs as target antigens. Additional studies in early cancer patients and high-risk individuals and the design of unique antigen panels for different cancers would help to determine whether multiple antigen mini-arrays for the detection of autoantibodies might contribute a clinically useful noninvasive approach to cancer detection and diagnosis.

Introduction
It is a well-established observation that autoimmunity can be associated with cancer, and one of the forms of its expression is the development of antibodies to autologous cellular antigens. One of the most extensively studied cancer-associated antigens is p53, a tumor suppressor protein. Autoantibodies to p53 in cancer were first reported in 1982 (1), and since then, there have been numerous reports confirming and extending this finding (reviewed in Ref. 2). The types of cellular proteins which induce autoantibody responses are quite varied and include oncogenic products, such as HER-2/neu and ras (3); cellular proteins, which shield mRNAs from natural physiological degradation, such as p62 (4) and CRD-BP (5); onconeural antigens in the paraneoplastic disorder syndromes (6, 7); and differentiation antigens, such as tyrosinase and the cancer/testis antigens (8).

Factors leading to the production of such autoantibodies are not completely understood, but the available data show that many of the target antigens are cellular proteins whose aberrant regulation could lead to tumorigenesis, such as p53, HER-2/neu, and ras, or are proteins whose dysregulation could have tumorigenic potential, including mRNA binding proteins, such as p62, and cell cycle control proteins, such as cyclin B1 (9, 10). A previous study showed that lung tumors contained several types of p53 gene mutations, including missense, stop codon, and frame shift mutations, but it was the missense mutations resulting in overexpression of protein with altered function and increased stability that correlated with antibody production (11). In the case of p62, which is a fetal protein absent in adult tissues, immunogenicity appears to be related to abnormal expression of p62 in tumor cells (12), and with the onconeural antigens in paraneoplastic neurological disorders, antibody responses are thought to be related to ectopic expression in tumor cells of normally neuron-restricted cellular proteins (6, 7). The immune system in certain cancer patients appears to have the capability of sensing these abnormalities, and we have proposed that autoantibodies might be regarded as reporters identifying aberrant cellular mechanisms in tumorigenesis (13).

Many investigators have been interested in the use of autoantibodies as serological markers for cancer diagnosis, especially because of the general absence of these autoantibodies in normal individuals and noncancer conditions. Enthusiasm for this approach has been tempered by low sensitivity. We showed recently (14) in a study using 777 sera from different types of cancer that autoantibodies to p62 were detected in 11.6 ± 1.15% and antibodies to Koc (15), another TAA,5 in 12.2 ± 1.17%. However, when the data were analyzed for

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5 The abbreviations used are: TAA, tumor-associated antigen; SS, Sjögren’s syndrome; dsDNA, double-strand DNA; NHS, normal human serum; SLE, systemic lupus erythematosus; HCC, hepatocellular carcinoma.
frequency of antibodies to either p62 or Koc, the frequency rose to 20.5 ± 1.45%. These initial observations were the basis for the current study using several hundred sera from six different types of cancer to determine reactivities to a mini-array of seven selected TAAs. We show that the frequency of antibodies to any individual antigen rarely exceeded 15–20%, but with the successive addition of antigens to the panel, there was stepwise increase in the percentage of positive reactors to between 44 and 68% against a combined panel of seven antigens. In addition, breast, lung, and prostate cancers showed separate and distinctive profiles of antibody responses, suggesting that tailor-made TAA panels could be developed for different cancers and that TAA mini-arrays might provide another approach to tumor detection and diagnosis.

Materials and Methods

Patients and Sera. The serum bank of the Tumor Cell Engineering Laboratory of Xiamen University, Fujian Province, People’s Republic of China, has a collection of sera from cancer patients which has been used for epidemiological and other studies, and this laboratory made sera available from 321 patients with different types of cancer, including 64 with breast cancer, 56 with lung cancer, 45 with colorectal cancer, 91 with gastric cancer, and 65 with HCC. As control, sera were obtained from 82 individuals from the same region who were having annual health examinations and had no evidence of malignancy. Sera from 206 prostate cancer patients treated at Loma Linda University Medical Center (Loma Linda, CA) were also available for this study. As comparison for the normal human sera from China, 264 sera from the San Diego area were available and came from a large epidemiological and genetic study of hemochromatosis, in which these “normal” sera were obtained from the nonhospitalized adult San Diego population (courtesy of Dr. E. Beutler, The Scripps Research Institute). In addition, as representative of patients with known immune reactivity to other autologous cellular antigens, sera from 62 patients with SLE and 41 patients with SS were available from the serum bank of the Autoimmune Disease Center (Scrpps) and were evaluated with the same immunoassays for their response to TAAs. This study was approved by the Institutional Review Boards of the respective academic centers.

Expression and Purification of Recombinant TAAs. Seven antigens, c-myc, p53, cyclin B1, p62, Koc, IMP1, and survivin were selected for the expression of recombinant proteins. Recombinant p62 has been expressed from a clone derived from a cDNA expression library by immunoscreening with antibody from a patient with HCC (4). p62 cDNA was subcloned into pET28a vector, producing a fusion protein with NH2-terminal 6x histidine and T7 epitope tags. The recombinant protein expressed in Escherichia coli BL21 (DE3) was purified using nickel column chromatography. Koc cDNA cloned in the pCDNA3 vector (15) was similarly subcloned to pET28a vector, and recombinant protein was expressed as above. IMP1 construct pCMV5-IMP1 was kindly provided by F.C. Nielsen (16) and p53 clone (p53SN3) by Yuxin Yin of Columbia University (New York, NY) and subcloned into pET28a vector for protein expression. cDNA from c-myc was amplified by PCR from human fetal liver tissue and survivin cDNA from human survivin expressed sequence tag clone (BQ258433) before subcloning in pET28a vector. Recombinant cyclin B1 had been prepared and used previously (9) and was isolated from a pGEX construct expressing cyclin B1 with glutathione S-transference fusion partner. The expression of adequate amounts of recombinant protein was examined in SDS-PAGE, and Coomassie Blue staining was used to determine that expression products of expected molecular sizes were produced. In addition, Western immunoblot analysis was used to confirm that the bands seen in SDS-PAGE were reactive with reference antibodies. The antibodies used were rabbit polyclonal anti-IMP1 from F.C. Nielsen (16), anti-Koc/IMP3 from F. Müller-Pillasch (15), which were raised against specific COOH-terminal peptides of the respective proteins, and anti-p62, which was raised against the full-length protein (4). Reactivities of p53, c-myc, and cyclin B1 were determined with monoclonal antibodies obtained from Oncogene Research Products (Boston, MA). Rabbit polyclonal antisurvivin antibody raised against the COOH-terminal peptide was obtained from ProSci, Inc. (Poway, CA).

ELISA. Purified recombinant proteins were diluted in PBS to a final concentration of 0.5 μg/ml, and 200 μl were pipetted into each well to coat Immulon 2 microtiter plates (Dynatech Laboratories, Alexandria, VA). All human sera were diluted 1:200, incubated with antigen-coated wells at room temperature for 90 min followed by washing with PBS, and developed with horseradish peroxidase-conjugated goat anti-human IgG (Caltag Laboratories, San Francisco, CA) using the substrate 2,2′-azinobis (3-ethylbenztheizoline-6-sulfonic acid) (Bayerwer Mannheim GmbH, Mannheim, Germany). Additional details of this procedure have been described previously (4, 14). Each serum sample was tested in duplicate, and the average A405 nm was used for data analysis.

The cutoff value designating positive reactions was conventionally chosen as the mean absorbance of the 82 normal Chinese human sera + 3 SD. Because several hundred test sera were analyzed at different time periods, each run of ELISA included 10 NHS samples and 2 positive control samples. The 10 NHS samples represented a range above and below the mean of the 82 Chinese normals, and the average value of the 10 normals was used in each run to normalize all absorbance values to the standard mean of 82 normals. In addition, all positive sera were confirmed with repeat testing, as were some negative sera. χ2 tests were initially used to determine whether the frequencies of positive sera in each cohort of cancer patients was significantly higher than in the normal cohort; statistical significance at α levels 0.05 and 0.01 are reported. Estimated sensitivities and specificities are given with associated SE.

Cancer sera were also tested against four cellular antigens: (a) dsDNA; (b) Sm; (c) SS-A/Ro; and (d) SS-B/La that are known antigenic targets of autoantibodies in the prototype autoimmune diseases, SLE, and SS (17). Although they are known to be autoantigens in systemic rheumatic diseases, cancer sera have not generally been evaluated for antibodies to these antigens, and this analysis could help to determine the extent of specificity of TAAs in cancer. Enzyme immunoassay kits from Helix Diagnostics (West Sacramento, CA) were used for these analyses.

Results

Frequency of Antibodies to Panel of TAAs and Range of Antibody Titers. Several parameters were considered to be important in the design of this study. The TAAs were selected to include oncogene product c-myc and tumor suppressor gene product p53. Cyclin B1 has been reported to be antigenic in humoral and cell-mediated immune responses in HCC and epithelial cell tumors, respectively (9, 10), p62 (4), Koc (15), and IMP1 (16) are three proteins containing similar RNA-binding motifs, and their mRNAs are either overexpressed in cancer (5, 15), or antibodies have been identified to the protein products (4, 14). Survivin is a cellular protein that is antiapo-
Diagnostic Mini-Array of Tumor-associated Antigens

above the mean for antibody to a TAA was taken as an absorbance reading
diseases, 62 patients with SLE, and 41 with SS. A positive test and as further controls representing noncancer autoimmune
United States, 264 normal human sera from the United States as the cancer patients, 206 prostate cancer patients from the
of seven TAAs using enzyme immunoassay. The sera that were
of these proteins were used as antigens, and sera from other autoimmune disorders were examined to establish that antibody reactivities observed were cancer specific and not attributable to nonspecific immunoreactivity.

Table 1 shows the frequency of autoantibodies to the panel of seven TAAs using enzyme immunoassay. The sera that were tested included the 321 patients from China with different types of cancer, 82 normal human sera from the same region in China as the cancer patients, 206 prostate cancer patients from the United States, 264 normal human sera from the United States and as further controls representing noncancer autoimmune diseases, 62 patients with SLE, and 41 with SS. A positive test for antibody to a TAA was taken as an absorbance reading above the mean + 3 SD of the 82 normal Chinese sera. As can be seen from Fig. 1, for as yet unknown reasons, the Chinese normal human sera generally had higher absorbance readings than the United States normal human sera. The decision to establish cutoff values using Chinese normal human sera was in part because the majority of cancer patients came from China and in part to be more rigorous in separating negative from positive data by using the higher set of “normal” values as cutoff.

In breast cancer, it was observed that there was a higher percentage of positive reactions to c-myc and Koc at P < 0.01, whereas positive reactions to p53, cyclin B1, p62, IMP1, and survivin were lower and not significantly different from normals. In lung cancer, positive reactions were found to c-myc, p53, cyclin B1, and p62 at P < 0.01 and to Koc and survivin at P < 0.05. Differences in the reactions of different cancers to TAAs were observed, and an interesting feature was that the highest positive reactions were not >26.8% against cyclin B1 in lung cancer and 25.2% against p62 in prostate cancer. Patients with lupus and SS contained occasional positive reactors like in normal human sera of both Chinese and United States origin.

Positive reactions to individual TAAs also showed considerable variation in the different cancer cohorts. Positive reactions to c-myc were detected in breast, lung, gastric, and liver cancer but were not different from normals in colorectal and prostate cancer. On the other hand, positive reactions with cyclin B1 were detected in lung, colorectal, gastric, liver, and prostate cancer but not in breast cancer. It is apparent from the data in Table 1 that different profiles of antibody reactivities could be observed using this panel of seven TAAs.

Many cancer sera showed absorbance readings several-fold above the cutoff, indicating that antibody responses in some patients were quite robust and not just mildly elevated (Fig. 1). As noted previously by many investigators, the likelihood that antibodies would be detected against any individual TAA did not reach levels of sensitivity, which would be useful as diagnostic biomarkers. However, against the collective panel of seven TAAs, the number of positive reactions increased to 43.8% for breast cancer and 67.9% for lung cancer. For the total of 321 Chinese cancer patients, the sensitivity of the seven TAA panel was 54.2%, and for the United States prostate cancer cohort, it was 46.1%. These data show the utility of the multiple antigen panel in increasing the sensitivity of antibody detection in cancer sera.

Stepwise Increase in Rate of Antibody Positivity with Successive Addition of Antigens. Table 2 shows that the sequential addition of antigens to the panel resulted in a stepwise increase in the number of positive reactions. This varied from one cancer to another and from antigen to antigen. The addition of p62 to IMP1 in the antigen panel increased the number of positive reactors in lung cancer (7.1–26.8%) and prostate cancer (8.7–31%), but there were lower increases in colorectal (13.3–17.8%) and gastric cancer (16.5–19.8%). In colorectal and prostate cancer, the addition of survivin did not further increase the number of positive reactors compared with other cancers. These observations suggest that for some cancers, putative TAAs like survivin may not be an optimum candidate for inclusion in a mini-array antigen panel and that specific panels should be developed for different cancers.

Preferential Reactivity of Some Cancer Sera with Certain Antigens but Absence of Coordinate Antibody Responses. The relative importance of each of the seven cancer-associated antigens for the different cancers is shown in Fig. 2. For breast cancer, it appears that c-myc and Koc antibodies are more frequently detected than other antibodies, but for lung cancer antibodies to cyclin B1, p62 and p53 are more frequently detected. In prostate cancer, there was higher frequency of antibodies to p62 and cyclin B1, and in HCC, anti-c-myc was predominant. There were fewer preferential reactivities in colorectal and gastric cancer. Fig. 3 presents an analysis of the data to determine whether there was coordinate or coupled expression of any two antibodies in the same serum. Because antibodies to c-myc and Koc were present in higher frequency in breast cancer than other combinations and antibodies to cyclin B1 and p62 were higher in lung cancer than antibodies to other TAAs, there was a possibility that these findings were related to coupled expression of some pairs of antibodies in the same
serum. If there was significant increased frequency of coordinate expression of two antibodies, this would be represented as higher frequency (percentage) in the bar graphs shown in Fig. 3. There appeared to be minor increases in frequencies of coexpressed antibodies, such as anti-c-myc and anti-p53 in breast cancer, anti-c-myc and anti-IMP1 in gastric cancer, and anti-c-myc and anti-IMP1 in HCC. These increases did not reach statistical significance, and in general, no clear coexpres-

### Table 2 Frequency of autoantibodies in relationship to number of antigen-antibody systems tested

<table>
<thead>
<tr>
<th>Antigen(s)</th>
<th>Type of cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Breast</td>
</tr>
<tr>
<td>IMP1</td>
<td>5 (7.8)</td>
</tr>
<tr>
<td>IMP1 or p62</td>
<td>8 (12.5)</td>
</tr>
<tr>
<td>IMP1 or p62 or Koc</td>
<td>14 (21.9)</td>
</tr>
<tr>
<td>IMP1 or p62 or Koc or p53</td>
<td>17 (26.6)</td>
</tr>
<tr>
<td>IMP1 or p62 or Koc or p53 or c-myc</td>
<td>25 (39.1)</td>
</tr>
<tr>
<td>IMP1 or p62 or Koc or p53 or c-myc or cyclin B1</td>
<td>26 (40.6)</td>
</tr>
<tr>
<td>IMP1 or p62 or Koc or p53 or c-myc or cyclin B1 or survivin</td>
<td>28 (43.8)</td>
</tr>
</tbody>
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*Numbers in parenthesis are percentage of positive reactors in that cancer category. Positive reactors are defined as above the mean of 82 NHS + 3 SD.*
sion of antibodies was observed. This finding supported the data in Table 2 showing that the sequential addition of antigens resulted in the detection of new antibodies, leading to the increased likelihood of detecting anti-TAA activity.

**Immunoreactivity of Cancer Sera with Other Cellular Autoantigens.** It was important to establish that autoantibodies to TAAs were not because of general immunoreactivity. Table 3 shows that reactivity with other cellular antigens, such as dsDNA, Sm, SS-A/Ro, and SS-B/La, was rarely found in cancer sera, but in SLE sera, they were in the expected and frequencies documented previously (17). There was reactivity in one or two cancer sera of 184 sera from five different types of cancers with a similar occasional reactivity in normal human sera, but these were unlike the higher frequencies observed in SLE sera.

**Discussion**

The most comprehensive studies on autoantibodies to TAAs have been on p53, and it was of interest to compare our data with those reported previously. Of the six different cancers in this study, the lowest positive rate was 4.9 ± 1.2% for prostate cancer, and the highest was 17.8 ± 2.13% for colorectal cancer. In a compilation of anti-p53 studies (2), the rate for prostate cancer was 2.7%, and it was 24.6% for colorectal cancer. The positive rates for breast, lung, gastric cancers, and HCC were intermediate between prostate and colorectal cancers in the review and this study. In our study, the positive rate for prostate cancer was not statistically different from the rate for normal human sera, and the same conclusion was found in previous studies, whereas the positive rates for breast, lung, colorectal, gastric cancers, and HCC were significantly different from normal controls in this and other studies (2, 20). This agreement with studies documented previously supports the accuracy of the immunoassay method used in this study.

This study points to an interesting feature of tumor immunity, that with an increasing number of antigens in a multiantigen immunoassay, the likelihood of detecting antibody in any cancer serum increased from ~15 to 26% when one antigen was used to 44–68% when seven antigens were used. Earlier studies that might have anticipated these results involved the use of a two-antigen panel (14) or was restricted to one type of cancer (21) in contrast to this study using seven known TAAs.
and several hundred patients with six different types of cancer. The sensitivity of the seven-antigen panel immunoassay system varied for different types of cancer. The factor or factors leading to these differences have not been examined, but from Fig. 2, it appeared that there was a stronger association between certain antigens and some cancers. In prostate cancer, p62 and cyclin B1 appear to be distinctly more reactive antigens than c-myc, p53, or survivin. In lung cancer, cyclin B1, p62, and p53 appear to be the more reactive antigens, whereas in breast cancer, they are c-myc and Koc. In HCC, c-myc stands out compared with other antigens which are about equal in antigenicity. A clear association of highly reactive antigens with colorectal and gastric cancer is not apparent. However, this initial study is based on seven TAAs, and the selection of antigens, although based on their known association with tumors, is by no means optimized and could be further refined with panels designed for greater specificity for certain types of cancer. There is support at the genetic level for this notion. Gene abnormalities, such as mutations or overexpression, tend to occur in combinations that vary from tissue to tissue (22), and if autoimmune responses represent immune system reactions to abnormal gene expression (13), one might expect differences in autoantibody profiles from one type of tissue cancer to another. Mini-arrays of TAAs could be constructed to include among others, several other known TAAs, such as HER-2/neu and ras, certain cell cycle proteins, and antigens involved in cell-mediated immune reactions, many of which might have concomitant humoral immune responses (8, 23).

The judicious selection of antigens to be included in panels or arrays of TAAs is extremely important because not all cellular proteins recognized as antigens by cancer sera are cancer specific. This was especially apparent in studies in HCC.

![Fig. 3](image-url)
where it was possible to analyze sera from the same HCC patients many months or years preceding malignancy when the patients had liver cirrhosis, chronic hepatitis, and autoimmune liver disease. Some of the autoantibodies identified with sera obtained when HCC was diagnosed were already present before the development of malignancy (24, 25), and these included antibodies against nucleolar antigens fibrillarin, nucleolus organizer region 90 protein, and B23, all of which are also recognized by antibodies from scleroderma and other autoimmune diseases. This feature has also been reported using SEREX technology (26), in which sera from colon cancer patients used in immunoscreening cDNA expression libraries identified several autoantigens associated previously with noncancer disorders, including hydroxy methylglutaryl-17, poly ADP ribose polymerase, and U1 small nuclear ribonucleoprotein, as well as cellular protein PINCH associated with aging and recognized by antibodies in normal human sera (27). The definition of TAA can probably be best determined by studies of significant numbers of cancer, as well as noncancer disorders.

Recently, an autoantigen array has been used for characterization of autoantibodies in patients with autoimmune rheumatic diseases, demonstrating potential applications, including the possibility of early diagnosis and characterization of specificity (28). Our study was not designed to show the predictive potential of autoantibodies as tumor markers but to report the novel finding of increasing sensitivity for detecting autoantibodies in cancer when larger panels of cancer-associated antigens are used. Nevertheless, cancer-associated antigen panels might conceivably be used for early detection of tumors in high-risk individuals. The appearance of antibodies to TAAs has been demonstrated in HCC in serum samples during antecedent liver disease (23, 29, 30). Anti-p53 antibodies were detected in two heavy smokers before clinical detection of lung cancer, and in 1 patient, early treatment resulted in good response, which correlated with total disappearance of p53 antibodies (31, 32). Anti-p53 antibodies were detected in workers exposed to vinyl chloride before clinical detection of angiosarcoma (33), and anti-HER-2neu antibodies can be detected in patients with early stage breast cancer (34). Anti-p53 antibodies have been detected in Barrett’s esophagitis (35), a condition often predating esophageal cancer and in beetal nut chewers with oral leukoplakia (36), a condition predating oral malignancy. Important future studies would be to determine whether mini-arrays of antibody markers might be useful in identifying early cancer in high-risk individuals. Furthermore, additional efforts should be aimed at increasing both the sensitivity and specificity of antibodies as cancer markers by expanding antigen arrays to include antigens which might be more selectively associated with some tumors and not with others.

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References


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